Environmental Health Perspectives
Vol. 93, 79–82, 1991

Molecular and Functional Analysis of Tumor-Suppressor Genes by Transfection

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The transformed and tumorigenic phenotype of H-*ras* transfected rat FE-8 cells can be suppressed by cell fusion with normal rat embryo fibroblasts. Transfection into FE-8 cells of DNA prepared from normal human placenta followed by selective elimination of tumorigenic transfected cell clones resulted in the isolation of phenotypically normal revertants. These cells exhibited a fibroblastlike, normal morphology; were anchorage-dependent; and were unable to proliferate in medium with reduced serum concentrations. Their tumorigenicity was also reduced. The suppressed phenotype has been transferred in a second transfection cycle. Human repetitive DNA sequences were detected in secondary transfectant DNA. A putative human suppressor gene, designated *NTS*-1, has been molecularly cloned. Reintroduction of cloned *NTS*-1 sequences into FE-8 cells resulted in suppression of the neoplastic phenotype in spite of a high *ras* expression.

Introduction

Genetic analysis of the neoplastic phenotype by somatic cell hybridization of tumor and normal cells has revealed that tumorigenic cells have accumulated genetic defects, resulting in the loss of gene activity essential for normal growth control. Tumorigenicity and transformed phenotypes are frequently abolished in tumor × normal hybrids (suppression of maligancy) (*1–3*). Several suppressor gene loci have been assigned to specific chromosomes in the human and rodent genomes. The suppressor genes active in tumor × normal somatic cell hybrids may be identical to those genes whose functional inactivation is associated with human malignancies; however, their molecular nature is not yet known. The function of tumor-suppressor genes, anti-oncogenes, or recessive genes is fundamentally different from that of cellular oncogenes in that they do not confer transformed properties upon normal cells (*1,4*). Rather, they appear to constrain the proliferation of transformed cells. We wished to study the phenomenon of tumor suppression in a cell system in which the neoplastic phenotype is triggered by a cellular oncogene frequently detected in human tumors. We established a functional assay for the molecular identification of DNA sequences associated with the induction of the suppressed phenotype. A putative tumor-suppressor gene, designated *NTS*-1, has been molecularly cloned.

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Suppression of Neoplastic Phenotype in H-*ras* Transformed Rat FE-8 Cells by Cell Fusion

Immortalized, nontumorigenic rat 208 F cells (deficient in hypoxanthine phosphoribosyltransferase activity, HPRT$^-$) were cotransfected with an activated human H-*ras* gene (EJ-*ras*) and the pSV*neo* gene. Transfectants were selected in medium containing G418 (Geneticin). One G418-resistant clone, FE-8, was chosen as the transformed parental cell line in subsequent cell fusion experiments. This clone contains four to five copies of the transfected *ras* gene and expresses high levels of p21. The cells exhibit a transformed morphology, form large colonies in semi-solid agar medium at a high efficiency, proliferate in medium with reduced serum concentrations, and initiate progressively enlarging tumors in newborn rats or nude mice without a detectable latency period.

HPRT-deficient, G418-resistant FE-8 cells were fused with normal rat embryo fibroblasts (REF), and somatic cell hybrids were selected in medium containing G418 and hypoxanthine, aminopterine, and thymidine (HAT additives). The hybrid clones resistant to HAT and G418 were isolated and analyzed for *ras* expression, the expression of transformed phenotypes *in vitro*, and for tumorigenicity. In spite of a high level of *ras* expres-

sion, FE-8 × REF somatic cell hybrids were nontumorigenic in newborn rats and no longer proliferated in semi-solid agar medium (*5*). Tumorigenicity reappeared in segregants with a reduced chromosome number derived from the suppressed hybrid clones. Similar results have been obtained in somatic cell hybrids involving *ras*-transformed Chinese hamster (CHEF) cells (*6*) or human EJ bladder carcinoma cells (*7*) and normal fibroblasts.

Suppression of Neoplastic Phenotype in H-*ras* Transformed FE-8 Cells by DNA Transfection

To identify and molecularly clone a suppressor gene that abolishes the neoplastic phenotype in *ras*-transformed cells, we have developed a functional assay based on DNA transfection. We assumed that an intact suppressor gene could be transfected into H-*ras* transformed FE-8 cells and exert its transformation-suppressing function even in the presence of the transforming gene. High molecular weight DNA from normal human placenta was cotransfected into FE-8 cells, together with the plasmid pY3 harboring a resistance gene encoding the hygromycin B phosphotransferase. Transfectants were selected in medium containing hygromycin B (HmB). A population of more than 6000 HmB-resistant, FE-8 transfectant clones was generated, in which the human donor genome was presumably represented several times. The population of transfected FE-8 cells was treated with the cardiac aminoglycoside ouabain in a second selection step. This drug has been shown to selectively kill *ras*-transformed cells in K^+-free medium, whereas nontransformed cells are able to survive the treatment (*8*). Colonies that had survived the ouabain selection were analyzed for the retention of a transforming *ras* gene, for the expression of the suppressed phenotype, and for the integration of human repetitive Alu sequences. Some of the selected transfectants appeared to be phenotypically unstable. Primary transfectants F4 and F9 retained a normal fibroblastlike morphology even after further maintenance in cell culture. These phenotypic revertants required high serum concentrations for growth, formed small colonies in semi-solid agar medium at a low efficiency, and their tumorigenicity in newborn rats was reduced. However, the two cell lines continued to express p21 at high levels.

The presence of a transforming *ras* gene in clones F4 and F9 was assayed by NIH 3T3 transfection. DNA prepared from F4 and F9 revertants was cotransfected into NIH 3T3 cells. Transfected NIH 3T3 cells were injected into nude mice. Tumors developed within a few weeks. The EJ-*ras* gene was detected in all such tumors. The partially suppressed phenotype was transferable in a second transfection cycle. DNA prepared from F4 and F9 cells was used as the donor DNA together with the pY3 plasmid in the second transfection cycle. Secondary transfected revertants were isolated after the two-step selection protocol. In a parallel experiment, F9 DNA was transfected without an added resistance gene. Transfectants were selected in medium containing HmB, and the ouabain treatment was omitted. This transfection experiment gave rise to a series of secondary transfectants, which retained a normal or intermediate morphology, and showed suppression of anchorage-independent growth and a reduced tumorigenicity.

We then attempted to molecularly clone the transfected human sequences from one of the revertant FE-8 clones. Clone FS9-7 harbored a single exogenous 18 kb BamHI fragment, as detected by hybridization to a human repetitive DNA probe. The same transfected DNA fragment was detectable by a pY3 probe. We concluded that the resistance gene used in the first transfection cycle and the human DNA fragment responsible for the expression of the suppressed phenotype must have been closely linked upon integration into the genome of tumorigenic FE-8 cells.

Molecular Cloning of the Candidate Suppressor Gene *NTS*-1

A recombinant phage library was constructed by inserting BamHI-digested FS9-7 DNA into the BamHI-site of the substitution phage vector EMBL 3. Six hundred thousand recombinant phages were screened for the presence of human repetitive DNA and for the hygromycin B phosphotransferase gene. Three recombinant phages, designated λ97-1, λ97-2, and λ97-3, were identified that harbored an 18 kb BamHI DNA-insert hybridizing to either probe. Recombinant phage DNAs were then transfected into FE-8 cells, and transfectants were selected in medium containing HmB. HmB-resistant clones were isolated after transfection of λ97-1 and λ97-3 DNA (Table 1). All HmB-resistant transfectants analyzed exhibited a low efficiency of cloning in soft agar medium. Individual colonies were small (less than 100 cells per colony) (Table 1). The tumorigenicity of these transfectants was reduced. We concluded from this experiment that the cloned 18 kb BamHI fragment

Table 1. Induction of the suppressed phenotype in H-*ras* transformed rat FE-8 cells: a comparison of transfection efficiencies.

Donor DNA	Calculated no. of colonies with suppressed phenotype/ μg DNA/10^6 transfected cells
Human placenta	0.02[a]
λ97-1	500[b]
λ97-2	—[c]
λ97-3	500[b]
pY3	< 0.003
Mock transfection (no DNA)	< 0.001

[a]Colonies identified after two-step selection protocol (see text).
[b]HmB-resistant colonies were directly analyzed for growth in soft agar medium. Ouabain selection was omitted.
[c]Transfection of λ97-2 DNA did not give rise to HmB-resistant colonies. Suppressive activity of this clone was not analyzed.

contained a functional human gene, capable of suppressing the transformed phenotype of H-*ras* transformed cells (*9*). The 18 kb BamHI fragment consists of DNA sequences encoding the hygromycin B phosphotransferase gene under the control of Moloney LTR sequences, a human Alu element, and unique human DNA sequences that are linked to rat DNA sequences derived from the recipient cell line.

Further transfection experiments using fragments derived from the original phage isolate, subcloned in a plasmid vector, have shown that a genomic DNA fragment, on which a unique human DNA sequence is linked to the resistance gene, is sufficient to confer the suppressed phenotype on FE-8 cells. Various transcripts related to *NTS*-1 have been detected in RNA of FE-8 transfectants. The sequence of the human genomic DNA element has been determined. Three open reading frames coding for polypeptides consisting of 42, 55, and 61 amino acids have been identified. These polypeptides are not homologous to any known protein sequence in the SWISSPROT data base. Experiments aimed at the further characterization of the putative suppressor gene, the isolation of the corresponding cDNA, analysis of its chromosomal localization, and of its expression pattern in normal and tumorigenic cells are in progress.

Suppression of Neoplastic Phenotype in Cells Transformed by Tyrosine Kinase Oncogenes

To determine whether the *NTS*-1 gene would counteract the expression of the neoplastic phenotype in cells transformed by oncogenes other than *ras*, we performed a series of cell fusion experiments. A revertant clone F9 ($HPRT^-$, HmB resistant) was fused with mouse NIH 3T3 or 208F rat cell lines ($HPRT^+$, HmB sensitive) containing the v-*src* or v-*fgr* oncogenes and with a mouse NIH 3T3 cell line ($HPRT^+$, HmB sensitive) transformed by polyoma virus. Somatic cell hybrids were selected in medium containing HmB, HAT additives, and semi-solid agar. The ratio of hybrid colonies proliferating in monolayer cultures to that of hybrids proliferating in semi-solid agar medium was determined. In control experiments, we generated somatic cell hybrids between the same transformed cell lines and normal rat 208F-3 cells or with tumorigenic FE-8Y cells ($HPRT^-$, HmB resistant). Cell fusions involving the cell lines transformed by tyrosine kinase oncogenes and both 208F-3 cells or F9 revertants harboring the *NTS*-1 gene yielded hybrid populations that were anchorage-dependent. In contrast to this, all hybrids involving Polyoma virus transformed cells continued to express the transformed phenotype. All hybrids between FE-8Y cells and transformed NIH 3T3 or 208F cells were transformed (*10*). These results suggest that the suppressor gene identified by transfection into H-*ras* transformed cells is capable of suppressing the neoplastic phenotype in cells transformed by tyrosine kinase oncogenes.

Conclusions

The transformed phenotype of H-*ras* transfected rat FE-8 cells can be suppressed by transected of DNA sequences derived from normal human cells. Human Alu sequences, detected in secondary transfected revertants, were used to identify the DNA sequence (*NTS*-1) associated with the expression of the suppressed phenotype. All revertants continued to express the transforming *ras* gene at high levels. Thus, the mechanism of suppression involves a process downstream of the initial interaction of the *ras* gene product with its target molecule(s). Understanding the mechanism of partial reversion in this cell system will require the characterization of the *NTS*-1 suppressor gene product, its subcellular localization, and expression pattern in normal and transformed cells. *NTS*-1 probes will be used to screen for changes in expression in suppressed somatic cell hybrids and in tumorigenic segregants as well as in human tumors.

Based on a similar functional assay, we have recently identified another human DNA sequence that is associated with the reversion of the transformed phenotype in transfectants derived from a spontaneously transformed Chinese hamster cell line (*11*; unpublished results). The candidate suppressor gene *NTS*-1 is not related to this sequence, nor is it related to the K-*rev*-1 gene (*12*), a suppressor gene identified by transfection of a human foreskin cDNA library into K-*ras* transformed NIH 3T3 cells. Thus, transfection into transformed cells of normal genomic DNA or of cDNAs isolated from normal cells has proved to be a useful approach to identify and clone candidate suppressor genes. These experiments, however, do not define a role for such genes in the process of human tumorigenesis. Rather, human tumors will have to be screened for genetic defects in the corresponding genes in order to search for a significant function in the initiation and progression of human neoplasia. The availability of functional molecular clones provides a unique tool to study suppressive effects in various cell systems and in cells transformed by oncogene products of different biochemical functions.

This work was initially supported by the Ludwig Institute for Cancer Research, Bern Branch. R. S. is the recipient of grant no. 31-25668 from the Swiss National Science Fund and of grant no. 392 from the Swiss Cancer League.

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